Increased 8-Isoprostane, a Marker of Oxidative Stress, in Exhaled Condensate of Asthma Patients

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Oxidative stress has an important role in the pathogenesis of asthma. 8-Isoprostane is a prostaglandin (PG)-F_2-like compound belonging to the F_2 isoprostane class that is produced *in vivo* by the free radical-catalyzed peroxidation of arachidonic acid. 8-Isoprostane is a biomarker of oxidative stress, and its concentration is increased in the bronchoalveolar lavage fluid of patients with interstitial lung diseases. We measured 8-isoprostane concentrations in exhaled breath condensate in healthy subjects and in patients with mild (steroid naive, n = 12), moderate (inhaled steroid treatment, n = 17), and severe asthma (oral steroid treatment, n = 15). We also measured exhaled carbon monoxide (CO) and nitric oxide (NO), which may also reflect oxidative stress in the airways. 8-Isoprostane was detectable in breath condensate of normal subjects (15.8 ± 1.6 pg/ml), and was increased in the breath condensate of patients with mild (33.7 ± 2.8, $p < 0.001$), moderate (38.3 ± 3.7 pg/ml, $p < 0.001$), and severe asthma (48.9 ± 5.0 pg/ml, $p < 0.001$). There was a positive correlation ($r = 0.68$, $p < 0.05$) of 8-isoprostane with NO, but not with CO, in the exhaled air of patients with mild asthma, but not in that of patients with moderate or severe asthma. There was no correlation between 8-isoprostane and lung function tests in any group of patients. Our study shows that oxidative stress is increased in asthmatic subjects as reflected by 8-isoprostane concentrations in breath condensate.
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Oxidative stress has an important role in the pathophysiology of asthma (1). Neutrophils and eosinophils from asthma patients produce increased amounts of oxygen free radicals when stimulated *in vitro* (2, 3). Antioxidant capacity is decreased in resting neutrophils from asthma patients (4), and oxygen radical production is increased in bronchoalveolar lavage (BAL) cells from patients with nocturnal asthma (5). Moreover, indicators of free radical activity are increased *in vivo* in adults and children with asthma (6, 7). There is evidence for an imbalance between oxidants and antioxidants in patients with stable and acute asthma (8). Recently, isoprostanes have been used to quantify oxidative stress *in vivo*. Measurements of these compounds in biologic fluid may therefore provide a quantitative index of oxidant stress *in vivo* (9). Isoprostanes are free radical-catalyzed products of arachidonic acid that are formed *in situ* in cell-membrane phospholipids, from which they are cleaved by phospholipase A_2 (10, 11). 8-Isoprostane, a member of the F_2 isoprostane class, has been detected in plasma and urine of humans (11, 12), and its levels are increased in smokers (13), in hepatorenal syndrome and acute paracetamol intoxication (14), and in scleroderma (15), all of which are pathophysiologic conditions in which oxidative stress is increased. Recently, we demonstrated an increase in 8-isoprostane in the bronchoalveolar lavage fluid (BALF) of patients with interstitial lung diseases (16). An increase in urinary levels of 8-isoprostane has recently also been demonstrated in patients with chronic obstructive pulmonary disease (COPD) (17). The aim of the present study was to investigate whether 8-isoprostane could be detected in breath condensate of asthma patients, and to compare its concentrations in these patients with those in healthy subjects. The use of breath condensate is a noninvasive means for collecting airway secretions.

METHODS

Patients

Ten healthy subjects and 12 patients with mild asthma, 17 with moderate asthma, and 15 with severe asthma were studied (Table 1). There was no significant difference in ages among the subject groups (Table 1). All study groups were matched for smoking habits, and abstinence from cigarette smoking was checked by urinary cotinine levels. Atopy was assessed by skin prick tests for common allergens. The diagnosis of bronchial asthma was based on the criteria of the American Thoracic Society (18). Severity of asthma was classified according to the National Institutes of Health/World Health Organization (NIH/WHO) guidelines (19). Briefly, subjects with mild asthma had symptoms twice a week or less often, with an $FEV_1 \geq 80\%$ predicted, and were taking regular medication but used inhaled β_2-agonists as needed for symptom relief. Subjects with moderate asthma had daily

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TABLE 1

PATIENT CHARACTERISTICS*

	Control Subjects	Mild Asthma	Moderate Asthma	Severe Asthma
Number	10	12	17	15
Age, yr	34.1 ± 2.8	27.8 ± 1.34	47 ± 5.15	38.9 ± 4.2
Sex, F/M	4/6	5/7	10/7	9/6
Smoking status				
Current smoker	0	0	0	0
Ex-smoker	1	1	2	1
Nonsmoker	9	11	15	14
FEV_1, % pred	113 ± 6.4	90 ± 3.6	71 ± 7.5†	49 ± 5.8†
Atopy	0	10	5	8
PC_{20}, mg/ml	> 8 mg/ml	4.3 ± 0.57	—	—

Definition of abbreviation: PC_{20} = concentration of methacholine provoking a 20% decrease in FEV_1.

* Data are expressed as mean ± SEM.

† $p < 0.01$ compared with normal subjects.

‡ $p < 0.001$ compared with normal subjects.

symptoms, used an inhaled short-acting β_2-agonist daily, had an FEV_1 between 60% and 80% predicted, and were taking regular inhaled glucocorticoids (budesonide: 0.4 to 3.2 mg, fluticasone propionate: 0.5 to 2 mg, or beclometasone: 1 to 2 mg). Subjects with severe asthma had continual symptoms, limited physical activity, frequent nocturnal asthma, and an $FEV_1 \leq 60\%$ predicted. Subjects with severe asthma were treated with oral prednisolone (4 to 50 mg/d) and inhaled steroids (fluticasone propionate: 1 to 4 mg/d, or budesonide: 0.8 to 4 mg/d).

Pulmonary Function

Pulmonary functions tests were performed within 2 wk after the measurement of exhaled markers. Spirometry was conducted with a dry spirometer (Vitalograph Ltd., Buckingham, UK), and the best value of three maneuvers was expressed as a percentage of the predicted value.

8-Isoprostane, NO, and CO were measured on the day of collection of the breath condensate sample.

Measurement of Exhaled 8-Isoprostane

Breath condensate samples were collected in a specially designed glass condensing chamber. The condensing chamber contained a glass double wall, and the inner side of the glass was cooled by ice. Breath condensate was collected between the two glass surfaces. Exhaled air entered and left the chamber through one-way valves at the inlet and at the outlet while the chamber was kept closed. After rinsing their mouths, subjects breathed tidally through a mouthpiece connected to the condenser for 15 min while wearing noseclips. Approximately 1 ml of condensate was stored at −70° C in a 2-ml sterile plastic tube (20).

8-Isoprostane concentrations in breath condensate were measured with a specific enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI). The assay was validated directly, by gas chromatography/mass spectrometry, to obtain a high correlation ($r = 0.95$) between added known amounts of 8-isoprostane and the concentration measured with the EIA. The antiserum used in the EIA has 100% cross-reactivity with 8-isoprostane and 0.2% cross-reactivity each with prostaglandin in $F_{2\alpha}$ ($PGF_{2\alpha}$), $PGF_{3\alpha}$, PGE_1, and PGE_2, and 0.1% cross-reactivity with 6-keto-$PGF_{1\alpha}$. The detection limit of the assay is 4 pg/ml. This assay kit has been used to measure 8-isoprostane concentrations in human BALF (16).

Exhaled NO Measurement

Exhaled NO was measured with a chemiluminescence analyzer (Model LR2000; Logan Research, Rochester, UK), sensitive to NO from 1 to 500 ppb by volume, and with a resolution of 0.3 ppb. The analyzer was designed for online recording of exhaled NO concentrations as previously described (21). It was calibrated with certified NO mixtures (90 ppb and 436 ppb) in nitrogen (BOC Special Gases, Guilford, UK). Measurements of exhaled NO were made by slow exhalation (5 to 6 L/min) from TLC for 20 to 30 s against a resistance (3 ± 0.4 mm Hg), to prevent nasal contamination.

Exhaled CO Measurement

Exhaled CO was measured with an electrochemical CO monitor sensitive to CO from 0 to 500 ppm by volume, which was adapted for online recording of CO concentration and integrated with the chemiluminescence analyzer to control exhalation parameters. The subjects exhaled slowly from functional VC with a constant flow (5 to 6 L/min) against a resistance (3 ± 0.4 mm Hg) over a period of 20 to 30 s into the analyzer. Two successive recordings were made, and the mean CO values were used in all calculations. Ambient CO levels were recorded before each measurement.

Statistical Analysis

One-way analysis of variance (ANOVA) with the Newman–Keuls test for multiple comparisons was used to compare groups. Linear regression analysis was used to assess the relationship between 8-isoprostane concentrations in breath condensate and exhaled gases. All data were expressed as mean ± SEM, and significance was defined as a value of $p < 0.05$.

RESULTS

Clinical data for healthy subjects and patients with asthma are summarized in Table 1. 8-Isoprostane concentrations were detectable (15.8 ± 1.6 pg/ml) in breath condensate of normal subjects, and were increased in subjects with mild (33.7 ± 2.8, pg/ml, $p < 0.01$), moderate (38.3 ± 3.7 pg/ml, $p < 0.001$), and severe asthma (49.1 ± 5.0 pg/ml, $p < 0.001$) (Figure 1). 8-Isoprostane levels were significantly increased in subjects with severe as compared with mild to moderate asthma (Figure 1).

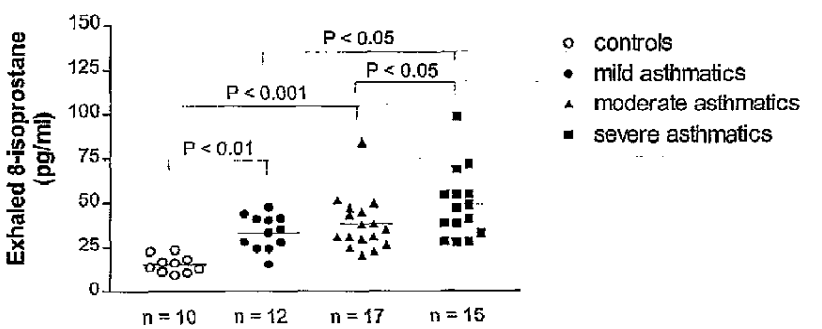

Figure 1. 8-Isoprostane concentrations in breath condensate in normal subjects and in patients with mild, moderate, and severe asthma. Data are expressed as mean ± SEM.

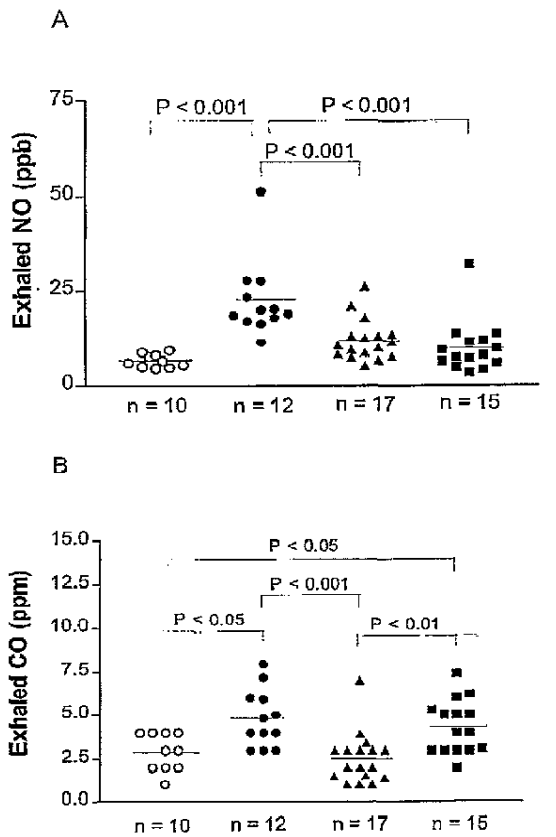

Figure 2. (*A*) NO concentrations in exhaled air of normal subjects and patients with mild, moderate, and severe asthma. (*B*) CO concentrations in exhaled air of normal subjects and patients with mild, moderate, and severe asthma. Data are expressed as mean ± SEM. Symbols are defined in Figure 1.

There was no correlation between 8-isoprostane level and age in healthy subjects. As part of the assessment of airway inflammation and oxidative stress, we also measured exhaled NO and CO in the same study groups. NO was significantly increased in subjects with mild asthma (22.6 ± 2.9 ppb, $p < 0.001$), but not in those with moderate (11.9 ± 1.3 ppb) or severe asthma (10.0 ± 1.8 ppb) as compared with normal subjects (6.5 ± 0.6 ppb) (Figure 2A). Exhaled NO levels in subjects with mild asthma were also significantly higher than those observed in subjects with moderate or severe asthma (Figure 2A). Exhaled CO was increased in subjects with mild (4.8 ± 0.5 ppm, $p < 0.05$) and severe asthma (4.3 ± 0.4 ppm, $p < 0.05$), but not in those with moderate asthma (2.5 ± 0.4 ppm) as compared with normal subjects (2.9 ± 0.3 ppm) (Figure 2B). Exhaled CO levels of subjects with severe asthma were significantly different from those observed for subjects with moderate asthma and the latter had lower exhaled CO levels than did subjects with mild asthma (Figure 2B).

In subjects with mild asthma, 8-isoprostane levels in breath condensate correlated with exhaled NO ($r = 0.68$, $p < 0.05$) (Figure 3). No correlation was found between 8-isoprostane concentrations in breath condensate and either exhaled CO or FEV_1.

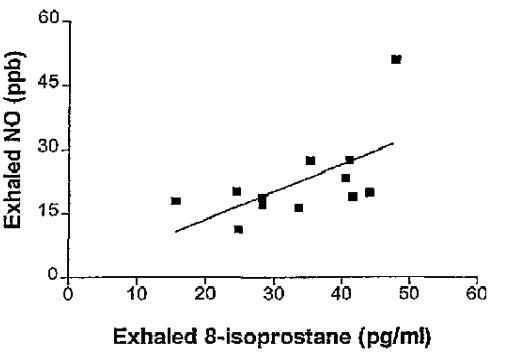

Figure 3. Correlation between NO levels in exhaled air and 8-isoprostane concentrations in breath condensate of subjects with mild asthma ($r = 0.68$, $p < 0.05$).

DISCUSSION

8-Isoprostane is the best characterized compound belonging to the F_2-isoprostanes, a group of stable $PGF_{2\alpha}$ isomers formed by free radical peroxidation of arachidonic acid independent of the action of cyclooxygenase (22). For this reason, 8-isoprostane has been considered an ideal marker for investigating the pathophysiology of oxidative injury. 8-Isoprostane may also be produced by cyclooxygenase (COX)-1 and COX-2 activity in some cells and tissues (23–25). However, despite its possible enzymatic synthesis, this isoprostane is still considered a reliable biomarker of lipid peroxidation caused by reactive oxygen species (26).

In this study, we showed that oxidative stress, as reflected by 8-isoprostane concentrations in breath condensate, is increased in asthmatic patients. As compared with those of healthy nonsmoking subjects, 8-isoprostane levels were approximately doubled in subjects with mild asthma and increased by about 3-fold in those with severe asthma. Subjects with moderate asthma had levels of 8-isoprostane comparable to those observed in subjects with mild asthma and lower than those of subjects with severe asthma, suggesting that inhaled steroids may to a certain extent control oxidative stress in these patients. Subjects with severe asthma showed the highest concentrations of 8-isoprostane in breath condensate, which reached statistical significance when compared with those of subjects with mild asthma. In the former subjects, 8-isoprostane concentrations in breath condensate seemed to be independent of steroid treatment. *In vitro*-induced 8-isoprostane production is decreased by dexamethasone via COX-2 inhibition (23). In subjects with severe asthma, high levels of 8-isoprostane despite systemic steroid treatment might suggest that *in vivo* this isoprostane is mainly derived from the nonenzymatic pathway. Our results are in keeping with those reported for patients with exacerbations of COPD, in whom steroid treatment before admission does not seem to affect the reduction in levels of oxidative stress observed at discharge (27). Although further studies are needed to quantify its possible enzymatic synthesis, 8-isoprostane seems to be a useful marker of oxidative stress in steroid-naïve patients and in severe asthma, in which a greater degree of inflammation is expected. This supports other evidence for increased oxidant stress in severe asthma, as demonstrated by an increase in exhaled pentane in acute exacerbations (28).

In keeping with previous studies (29–31), NO and CO levels in exhaled air in our study were increased in subjects with

mild asthma, but were not different from those of normal subjects or those with moderate asthma, showing that these biomarkers of oxidative stress are sensitive to inhaled steroid treatment. In this study we measured for the first time the levels of NO and CO in the exhaled air of patients with severe asthma. In these patients, NO levels were similar to those observed in normal subjects, suggesting a possible dependence on steroid treatment. Inhaled steroids decrease inducible NO synthase (iNOS) expression in the airway epithelium of asthma patients (32); pretreatment with iNOS inhibitors also decreases exhaled NO in asthma patients (33). Alternatively, the lower exhaled NO levels in the patients with severe asthma in our study could have been due to the interaction of NO with other oxidants, such as superoxide anion. This may occur to a greater extent with greater oxidative stress in patients with severe asthma, thus producing products such as peroxinitrite and so reducing the levels of exhaled NO. In patients with severe asthma, exhaled CO was increased compared with that of normal subjects, showing a pattern similar to that seen with 8-isoprostane. This could reflect the relative lack of efficacy of steroid treatment in controlling higher levels of oxidative stress in patients with severe asthma. In view of the protective role of hemeoxygenase activation, an increase in CO production may represent a homeostatic mechanism in pathologic conditions characterized by high levels of oxidative stress (34). In patients with mild asthma, 8-isoprostane concentrations in breath condensate were found to correlate with NO in exhaled air, suggesting that NO released from inflammatory cells is increased by some conditions that lead to the production of this isoprostane (35).

In conclusion, we have shown that 8-isoprostane is detectable in breath condensate of healthy subjects, and that its levels are increased in asthma patients. Oxidative stress, as reflected by 8-isoprostane concentrations in breath condensate, is progressively increased with the severity of asthma. Although controlled studies are needed to establish how glucocorticoids modulate 8-isoprostane concentrations, this biomarker seems to show a certain degree of resistance to steroid treatment. Considering this and the relative lack of reliable *in vivo* indices of oxidative stress, 8-isoprostane seems to be a promising biomarker of the severity of asthma.

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